REMARKS

Favorable reconsideration, reexamination, and allowance of the present patent application are respectfully requested in view of the foregoing amendments and the following remarks. No new matter is added by the foregoing amendments. Applicants greatly appreciate the withdrawal of the rejections as indicated by the Examiner on page 2 of the Office Action.

Rejection under 35 U.S.C. § 112, second paragraph

In the Office Action, beginning at page 2, Claims 7-9, 12 and 13 were rejected under 35 U.S.C. § 112, second paragraph, as reciting subject matters that allegedly are indefinite. Applicant respectfully requests reconsideration of this rejection.

Claims 7 and 12 have been amended to recite the term "is" in place of "comprises." Claim 9 has been amended to recite the terms "consisting of" in place of "comprising." Claims 8 and 13 have been cancelled without prejudice.

For at least the foregoing reasons, Applicant respectfully submits that Claims 7 and 12 fully comply with 35 U.S.C. § 112, second paragraph, and therefore respectfully requests withdrawal of the rejection thereof under 35 U.S.C. § 112.

Rejection under 35 U.S.C. § 112, first paragraph

In the Office Action, beginning at page 3, Claims 1 and 6 were rejected under 35 U.S.C. § 112, first paragraph, as reciting subject matters that allegedly contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Applicant respectfully requests reconsideration of this rejection.

Applicants respectfully assert that the Examiner cannot *per se* require recitation of

the nucleotide sequence of a known gene in the specification, or the claims, to adequately describe the claimed invention. An applicant is permitted to be his or her own lexicographer. The *E.coli rmf* gene and amino acid sequences are well-described in the prior art and in the specification. The endogenous gene from *E. coli* has been reported in the literature and is a known sequence, although perhaps with some natural variation. Applicants have defined, consistent with the prior art definition, the *E. coli rmf* gene as being the sequence shown in the prior art. The Examiner states that the specification does not provide the specfic SEQ ID NO: of the E. coli RMF gene, but only cites to literature references. This does not indicate that the *E.coli rmf* gene is not described. This issue is directly on point with the recent CAFC decision in *Capon v. Eshhar* (03-1480, -1481), wherein the court stated that a sequence which is known in the prior art is not required to be repeated in the specification. The court reasoned that applicants are not required, and are actually specifically discouraged from reciting in the specification that which is well-known in the art. Obviously, if the court does not require recitation of the well-known sequence in the specification, there is no reason it needs to be recited in the claims.

The *E. coli* RMF gene as well as an upstream sequence containing expression control sequences was clearly known in the art at the time of the invention. As stated in the previous response, figure 1B of Yamagishi et al. *EMBO J.* (1993) 12: 625-630, which is cited in the specification and pointed to by the Examiner, shows the deduced promoter sequence as well as the sequence of the upstream region.

Furthermore, the invention is NOT the well-known *E.coli rmf* gene already taught in the prior art, but a method of producing L-amino acids by disrupting the endogenous *E. coli* rmf gene which results a novel and unobvious increase in amino acid production and excretion by the *Escherchia coli* cell. Therefore, applicants have clearly and adequately described their invention such that one of skill in the art would recognize that applicants were in possession of the invention at the time of the invention.

The Office Action states that many *E.coli* rmf genes with widely differing structural, chemical, and physical properties are included, however, it is not clear that the claim is being read in light of the specification and prior art. The *E.coli rmf* gene has been well-characterized in the prior art, and some variation is possible to exist among cell types, even though they belong to the same genus and species. However, such variation does not represent "widely differing structural, chemical, and physical properties" but represents natural variation taught in the prior art and easily discernable as such by a person of ordinary skill in the art. The Examiner cannot read the claims in a vacuum, and when the claims are read in light of the prior art and specification, it is clear that the rmf gene and protein are well-characterized and well-described entities.

The Office Action states on page 4 that there is no disclosed nucleotide sequence and structure that is common to the genus of *E. coli rmf* genes. In this statement, it is clear the Examiner is not reading the claims in light of the disclosed and well-characterized nucleotide sequence which is the rmf gene taught in the prior art.

In regard to the assertion that other types of amino acids, such as a liphatic, aromatic, hydroxylic, etc., are not shown as being increased by the methods of the invention, Applicants continue to respectfully assert that two exemplary amino acids are sufficient to describe the genus.

The mechanism of the invention is that bacterial growth in a stationary phase is increased by disruption of the *rmf* gene, and thereby L-amino acid-productivity is increased (see page 3, first incomplete paragraph of the specification). Furthermore, Examples 2 and 3 of the specification show that growth of the *rmf* gene-disrupted strain WC196Δrmf is improved as compared to the parent strain WC196 (see Fig. 1 and Fig. 4). Similar results were shown in the Rule 132 declaration file in response to the previous office action (see Fig. 1(A)). Also, similar results are also expected such that rapid growth of a bacterium leads to the improvement of the production rate of L-amino acids. Therefore, two exemplary amino acids are considered sufficient to describe the claimed

invention.

For at least the foregoing reasons, Applicant respectfully submits that Claims 1 and 6 fully comply with 35 U.S.C. § 112, first paragraph, and therefore respectfully requests withdrawal of the rejection thereof under 35 U.S.C. § 112.

In the Office Action, beginning at page 4, Claims 7, 8, 12 and 13 were rejected under 35 U.S.C. § 112, first paragraph, as reciting subject matters that allegedly which are not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicant respectfully requests reconsideration of this rejection.

In regard to the strain WC196Δrmf, this strain can be prepared from the deposited strain WC196 (AJ13069) according to the description in the specification, and therefore, a deposit of this strain is not required for enablement and/or description of the invention.

The strain WC196 (AJ13069) has been deposited under the Budapest Treaty. A copy of the receipt of the deposit is attached to this response. The strain WC196Δrmf can be constructed by disruption of the *rmf* gene according to well-known techniques as described in, for example, Example 2 of the specification. As a temperature sensitive plasmid used for disruption of the *rmf* gene, pMAN997 is used in Example 2 of the specification. The plasmid pMAN997 can be prepared from known plasmids pMAN031 and pUC19 according to WO99/03988. pMAN031 is described in J. Bacteriol., 162, 1196-1202 (1985) (copy enclosed). In particular, please note the scheme on page 1198. pUC19 can be purchased from, for example, Takara Shuzo.

For at least the foregoing reasons, Applicant respectfully submits that Claims 7 and 12 fully comply with 35 U.S.C. § 112, first paragraph, and therefore respectfully requests withdrawal of the rejection thereof under 35 U.S.C. § 112.

Conclusion

For at least the foregoing reasons, Applicant respectfully submits that the present patent application is in condition for allowance. An early indication of the allowability of the present patent application is therefore respectfully solicited.

If Examiner Fronda believes that a telephone conference with the undersigned would expedite passage of the present patent application to issue, he is invited to call on the number below.

It is not believed that extensions of time are required, beyond those that may otherwise be provided for in accompanying documents. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the undersigned respectfully requests that she be contacted immediately.

Respectfully submitted,

By: _____

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Date: October 14, 2005

INTERNATIONAL FORM

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

DEPOSITOR NAME: Ajinomoto Co., Inc.
President, Shunsuke INAMORI
ADDRESS: 15-1, Kyobashi 1-chome, Chuo-ku,
Tokyo 104 Japan

I. Indication of a Microorganism	
(Indication by depositor to specify the microorganism) Escherichia coli AJ13069	(Deposit Number) FERM BP-5252
II. Scientific nature and a position by taxonomy	
The microorganism specified in the column I was attached to documents in which following matters were described. ☐ scientific nature ■ a position by taxonomy	
III. Receipt and Acceptance	
Our international depositary authority accepts the microorganism specified in the column I received on December 6, 1994 (original deposit date).	
IV. Receipt of transfer request	
Our international depositary authority received the microorganism specified in the column I on December 6, 1994 (original deposit date). And we accepted a transfer request to deposit under Budapest Treaty from the original deposit on September 29, 1995. (Transfer from FERM P-14690 deposited on December 6, 1994)	
V. International depositary authority	
NAME: National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology Michio OISHI, Ph.D., DIRECTOR GENERAL ADDRESS: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305 Japan (sealed) Dated September 29, 1995	

国際様式 INTERNATIONAL FORM

〔特許手続上の微生物の寄託の国際的承認に関するブダペスト条約〕

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

下記国際寄託当局によって規則7．1に従い発行される

原寄託についての受託証

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

寄託者 氏名（名称） 味の素株式会社 代表取締役社長 稲森 俊介 殿

あて名 〒104 東京都中央区京橋一丁目15番1号

I．微生物の表示

（寄託者が付した識別のための表示）	（受託番号）
Escherichia coli AJ13069	FERM BP- 5252

II．科学的性質及び分類学上の位置

I欄の微生物には、次の事項を記載した文書が添付されていた。

☐ 科学的性質

☒ 分類学上の位置

III．受領及び受託

本国際寄託当局は、平成 6年 12月 6日（原寄託日）に受領したI欄の微生物を受託する。

IV．移管請求の受領

本国際寄託当局は、平成 6年 12月 6日（原寄託日）にI欄の微生物を受領した。
そして、平成 7年 9月29日に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。
（平成 6年 12月 6日に寄託された微工研菌寄第 P- 14690 号より移管）

V．国際寄託当局

通商産業省工業技術院生命工学工業技術研究所

名称： National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology

所長 大石道夫

Michio Oishi, Ph. D., DIRECTOR GENERAL.

あて名：日本国茨城県つくば市東1丁目1番3号（郵便番号305）
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平成 7年（1995） 9月 29日

JOURNAL OF BACTERIOLOGY, June 1985, p. 1196-1202
0021-9193/85/061196-07$02.00/0

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Construction and Characterization of a Deletion Mutant Lacking *micF*, a Proposed Regulatory Gene for OmpF Synthesis in *Escherichia coli*

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A method is presented for the construction of a deletion mutant lacking the *micF* gene, which has been proposed to negatively regulate expression of the *ompF* gene. The method includes (i) construction of a temperature-sensitive plasmid containing a chromosomal fragment that carries both flanking regions of the *micF* gene but does not carry *micF* itself and (ii) replacement of the corresponding chromosomal domain with the fragment. The method is applicable to construction of a deletion mutant for any *Escherichia coli* chromosomal gene provided that it is dispensable. The *micF* deletion was confirmed by genetic and biochemical tests, including nucleotide sequence analysis. *ompF* expression in the *micF* deletion mutant thus constructed was normally regulated and was not enhanced. When *micF* was cloned into a high-copy-number plasmid it repressed *ompF* gene expression, whereas when cloned into a low-copy-number plasmid it did not. From these results, it is concluded that a single copy of the *micF* gene on the *E. coli* chromosome does not play a critical role in *ompF* gene expression.

The outer membrane of *Escherichia coli* contains two major porin proteins, OmpF and OmpC, that serve as channels for the passive diffusion of small hydrophilic molecules (25) and as constituents stabilizing the cell surface (26). These proteins are encoded by the *ompF* and *ompC* genes, respectively. Both genes have been cloned (19, 23), and their total nucleotide sequences have been determined (13, 20). These studies revealed high homology in the deduced amino acid sequences as well as the nucleotide sequences in the coding regions between *ompF* and *ompC* (20). On the other hand, the promoter structures of these genes are considerably different.

Although the functional and structural properties of the two proteins are similar, the expression of the genes is regulated in opposite directions by the medium osmolarity (15, 31). As the osmolarity increases, OmpF synthesis is depressed, with a concomitant increase in OmpC production. Studies with hybrid genes, such as the *ompF-lacZ* and *ompC-lacZ* fusions (9, 10), and chimeric genes between the *ompF* and *ompC* genes (17) revealed that the promoter region is primarily responsible for osmoregulation. Expression of *ompF* and *ompC* is also regulated by the *ompR* and *envZ* genes in the *ompB* operon (7, 11, 24, 30). The OmpR protein serves as a positive regulator for the *ompF* and *ompC* expression; the role of *envZ* is less clear.

Recently, Mizuno et al. (21, 22) found a third regulatory gene, *micF*, which codes for a small RNA molecule. This gene is located to the right upstream of *ompC*, and its transcription direction is opposite that of the *ompC* gene. *micF* gene expression is under the control of the *ompR* operon in the same manner as the *ompC* gene is. The primary structure of the *micF* RNA is complementary to the 5'-end region of the *ompF* mRNA, and when cloned into pBR322 the gene inhibits the production of the OmpF protein. Based on these facts, *micF* RNA was proposed to inhibit translation of the *ompF* mRNA by hybridizing with it and to play a critical role in the osmoregulation of OmpF and OmpC synthesis.

For a critical study on the role of *micF* in expression of the *ompF* and *ompC* genes, we constructed a *micF* deletion mutant. The method includes (i) construction of a plasmid containing a chromosomal fragment that carries both flanking regions of the *micF* gene but does not carry the *micF* gene itself and (ii) replacement of the corresponding chromosomal domain with the fragment. *ompF* expression in the mutant was osmoregulated normally and was not enhanced, suggesting that one copy of the *micF* gene does not play a critical role in osmoregulation. We also examined the effect of the copy number of *micF* on *ompF* expression. Although when *micF* was cloned into a high-copy-number plasmid it inhibited OmpF synthesis, when it was cloned into a low-copy-number plasmid it did not. Based on these observations, the role of the *micF* gene is discussed.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The *E. coli* K-12 strains, bacteriophages, and plasmids used in this work are listed in Table 1.

Media. Expression of the *ompF* and *ompC* genes was studied by using medium A supplemented with different concentrations of sucrose as described previously (15). The sensitivity of the cells to phage TuIb was tested by cross-streaking on medium A with 15% (wt/vol) sucrose. Transformation experiments were carried out in 1% tryptone–0.5% yeast extract. When required, ampicillin, chloramphenicol, and kanamycin were added at concentrations of 50, 25, and 30 μg/ml, respectively. For solid cultivation, the medium was supplemented with 1.5% agar.

Cell envelope preparation and polyacrylamide gel electrophoresis. Preparation of cell envelopes, solubilization with Triton X-100, and urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described previously (24).

DNA techniques. Restriction endonucleases, bacteriophage T4 ligase, exonuclease *Bal* 31, S1 nuclease, *Hin*dIII

* Corresponding author.

TABLE 1. Bacteria, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant properties	Reference or source
E. coli K-12		
MC4100	F⁻ *ΔlacU169 araD rpsL relA thi fibB*	4
MH760 *recA*	F⁻ *ΔlacU169 araD rpsL relA thi fibB ompR472 gyrA recA*	12
HO201	F⁻ *thi rel rpsL mal* λ^r	32
HO201 *ompC*	*ompC* derivative of HO201	27
YO160 *recA*	F⁻ *thi rel rpsL ompC envZ Φ(ompC-lacZ) recA*	17
CE1036 *recA*	F⁻ *thi lacY galK mil xyl ara rpsL supE ompC* T6^r *recA*	17
SM3001	MC4100 Δ*micF1*	This study
SM3002	Δ*micF1* transductant of HO201; donor, SM3001	This study
Bacteriophages		
Tulb	Receptor; OmpC and lipopolysaccharide	6
P1*kc*	Used for generalized transduction	Our laboratory stock
Plasmids		
pMAN002	Cm^r; vector, pACYC184; cloned gene, *ompC micF*	17
pMAN006	Ap^r; vector, pKEN403; cloned gene, *ompC micF*	17
pMAN005	Same as pMAN006 except for direction of the cloned fragment	This study
pSY343	Km^r	33
pEL3	Ap^r; temperature-sensitive replicon	1
pKM004	Ap^r; vector, pBR322; cloned gene, *lpp* promoter-controlled *lacZ-lacY* operon	21
Plasmid III	Ap^r; vector, pKM005; cloned gene, *micF*	22
pKEN403	Ap^r Km^r; replication origin derived from pSC101	K. Nakamura
pACYC184	Cm^r Tc^r	5
pBR322	Ap^r Tc^r	2

linker (dCAAGCTTG), and *Bam*HI linker (dCGGATCCG) were obtained from Takara Shuzo Co. The conditions used for digestion with these nucleases were those proposed by the manufacturer. [γ -^{32}P]ATP labeling at the 5′ ends of DNA fragments for hybridization and sequencing and DNA sequencing were carried out as described by Maxam and Gilbert (18). Southern transfer was carried out as described previously (17), and the blot was hybridized with a ^{32}P-labeled probe (10^6 cpm/ml) at 37°C in the presence of a deionized solution of 50% formamide-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Other general manipulations such as preparation of plasmid and chromosomal DNAs, digestion with restriction endonucleases, ligation, and transformation were performed as described previously (17).

RESULTS

Construction of plasmid pMAN036. Deletion of the *micF* region from the chromosome was carried out in two steps: (i) construction of a plasmid containing a chromosomal DNA fragment that carries both flanking regions of the *micF* gene but does not carry the *micF* gene itself, and (ii) replacement of the corresponding chromosomal domain with the fragment. For this purpose, pMAN036 was first constructed. The detailed procedure for its construction is shown in Fig. 1. Structures of the individual plasmids shown in Fig. 1 were confirmed by DNA restriction analysis.

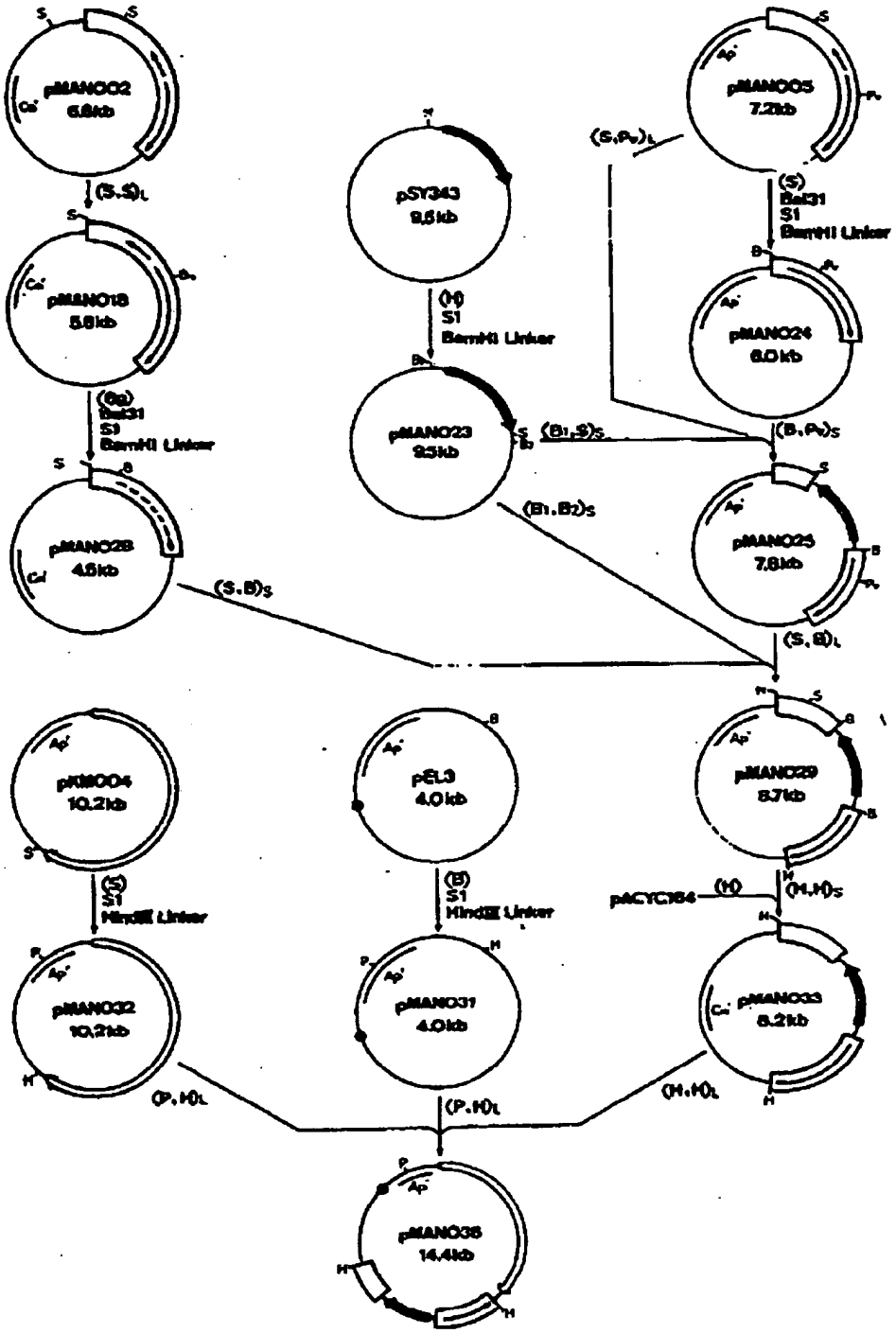
pMAN005, pMAN002, and pSY343 were used as starting plasmids. Deletion by *Bal* 31 digestion was performed for pMAN005 and pMAN018 to construct pMAN024 and pMAN028, respectively. For pMAN024, digestion was initiated at the *Sal*I site located about 550 nucleotides downstream from the possible *micF* termination signal (22) and reached the *micF* promoter region. For pMAN028, digestion was initiated at the *Bgl*II site located near the promoter-proximal end of *ompC* and reached the promoter-distal end of *micF*. The extent of the deletion was determined by size analysis of the *Bam*HI-*Bgl*II fragment for pMAN024 and of the *Sal*I-*Bam*HI fragment for pMAN028.

From these plasmids was constructed pMAN029. Since we wanted to construct other plasmids together with pMAN029, the process (Fig. 1) was somewhat complex. Plasmid pMAN029 carries both flanking regions of the *micF* gene but carries the kanamycin resistance (Km^r) gene in place of *micF*. The structure of the plasmid was confirmed by DNA restriction analyses and the presence of both the Km^r and *ompC* genes. The vector domain of pMAN029 was then replaced by that of pACYC184 carrying the chloramphenicol resistance (Cm^r) gene to facilitate the selection of pMAN036. Finally, from pMAN033, pKM004, and pEL3 was constructed pMAN036. This plasmid carries (i) the *ompC-micF* region of the chromosomal DNA in which the *micF* gene is replaced by the Km^r gene, (ii) a temperature-sensitive replicon, and (iii) the *lpp* promoter-controlled *lacZ-lacY* operon. All of these characteristics except the absence of *micF* were confirmed biochemically. The structure of pMAN036 was also confirmed by DNA restriction analysis of the entire plasmid.

Construction of a chromosomal mutant with *micF* deletion. A *micF* deletion mutant was constructed by replacing the *micF-ompC* region of the chromosome with the Km^r-*ompC* region of pMAN036 via homologous recombination (Fig. 2). Use of the temperature-sensitive replicon-carrying plasmid enabled us to isolate transformants in which the plasmid had integrated into the chromosome. Use of the Km^r and *lac* genes arranged as in pMAN036 facilitated selection of the *micF* deletion mutant.

E. coli MC4100 (Δ*lac*) was transformed with pMAN036 and grown at 30°C for 2 h. It was further incubated on lactose-MacConkey plates containing kanamycin (30 μg/ml) at 42°C overnight. Since pMAN036 has the temperature-sensitive replicon, the transformants should appear as Km^r colonies at 42°C only after incorporation of the plasmid into the chromosome (1, 8). Most of the transformants appeared as red colonies (Km^r Lac$^+$), indicating that the plasmid had been integrated into the chromosome by a single crossover at either region A or B (Fig. 2). About 1% of the transformants appeared as white colonies (Km^r Lac$^-$). Provided that the events shown in Fig. 2 did take place in the transformants, they are the most probable candidates for chromosomal mutants carrying the *micF* deletion.

Determination of the genomic structure of SM3001, a *micF* deletion mutant. Three experiments were performed to confirm the gene structure of the relevant region of strain SM3001, one of the presumed *micF* deletion mutants. First, P1 transduction was carried out with strain SM3001 as the donor and strain HO201 *ompC* as the recipient. All of the 300 Km^r transductants isolated were OmpC$^+$, indicating that the


pMANO02
6.6kb
(S,S)L
pMANO18
5.6kb
(Ba)
Bal31
S1
BamHI Linker
pMANO28
4.5kb
(S,B)S
pSY343
9.6kb
(H)
S1
BamHI Linker
pMANO23
9.5kb
(B1,S)S
(B1,B2)S
pMANO05
7.2kb
(S,Pv)L
(S)
Bal31
S1
BamHI Linker
pMANO24
6.0kb
(B,Pv)S
pMANO25
7.8kb
(S,B)L
pKH004
10.2kb
(S)
S1
HindIII Linker
pMANO32
10.2kb
(P,H)L
pEL3
4.0kb
(H)
S1
HindIII Linker
pMANO31
4.0kb
(P,H)L
pMANO29
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(H)
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(H,H)L
pMANO36
14.4kb

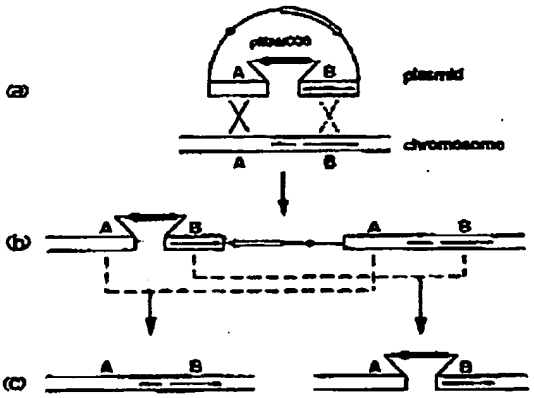

FIG. 2. Replacement of the *micF-ompC* region of the chromosome with the *micF*-deleted corresponding region in pMAN036. Strain MC4100, a Δ*lac* strain, was transformed with pMAN036 (Fig. 1) and incubated at 42°C on lactose-MacConkey plates containing kanamycin. Plasmid integration into the chromosome by homologous recombination takes place at either region A or region B (a). Transformants having the plasmid in the chromosome are Km^r Lac^+. In the case of recombination at region A, subsequent plasmid segregation from the chromosome by homologous recombination takes place at either of the regions indicated by dotted lines in (b). This results in one of the chromosomal structures shown in (c). The segregated plasmids are lost in the cells because of the inability to replicate autonomously at 42°C. Recombination for plasmid segregation at region A gives the same chromosomal structure as that of an untransformed cell, whereas recombination at region B results in replacement of the *micF* gene with the Km^r gene. This *micF* deletion mutant is detected as a white colony (Lac^-) on a lactose-MacConkey plate containing kanamycin. When recombination for plasmid integration takes place at region B (a), the subsequent process is principally the same. The symbols used are described in the legend to Fig. 1.

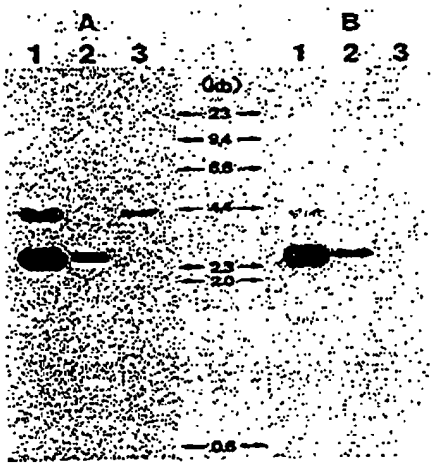

FIG. 3. Southern blot analyses of the *micF* chromosomal deletion. A mixture of pMAN002 and pMAN033 (lanes 1) and chromosomal DNA from strains MC4100 (lanes 2) and SM3001 (lanes 3) was digested with *Hin*dIII and analyzed as described in the text. The 2.7-kb fragment containing the *micF-ompC* region from pMAN002 (A) and the 120-bp fragment containing a larger part of the *micF* gene (Fig. 4) (B) were used as probes. The *Hin*dIII fragments of bacteriophage λ DNA were used for standardization of the base length.

Km^r gene is very closely linked to the *ompC* gene on the chromosome.

In the second experiment, the chromosomal DNA of strain SM3001 was analyzed by Southern hybridization to confirm the absence of the *micF* gene. When the 2.7-kilobase (kb) *Hin*dIII fragment carrying the *ompC-micF* region isolated from pMAN002 was used as a probe, hybridization with the *Hin*dIII digest of the wild-type chromosomal DNA took place at the position of 2.7 kb as expected (Fig. 3A, lane 2). On the other hand, the probe hybridized with the *Hin*dIII digest of the strain SM3001 chromosomal DNA at the position of 4.2 kb (Fig. 3A, lane 3). This size was the same as that of the *Hin*dIII fragment of pMAN033 that carries the *ompC* region in which the *micF* gene has been replaced by the Km^r gene. No other bands were observed on Southern blotting. Hybridization analysis was also performed with a 120-base-pair (bp) fragment carrying a larger part of the *micF* gene. The probe hybridized with the *Hin*dIII digest of strain MC4100 chromosomal DNA at the position of 2.7 kb, but no significant hybridization was observed with the *Hin*dIII digest of strain SM3001 chromosomal DNA (Fig. 3B). Weak hybridization took place at the position of 4.2 kb. As revealed later by DNA sequencing analysis, the 4.2-kb *Hin*dIII fragment of strain SM3001 still possessed a small portion of the promoter-distal end of the *micF* gene that shares 17 nucleotides with the 120-bp probe (Fig. 4). Taken together, these results demonstrate that (i) the *micF* gene is almost fully deleted and replaced by the Km^r gene in the mutant strain SM3001 chromosome and (ii) the wild-type *E. coli* chromosome possesses only one copy of *micF*.

In the third experiment, the relevant region of strain SM3001 chromosomal DNA was recloned into a plasmid, and the nucleotide sequence was determined. The chromosomal DNA of strain SM3001 was digested with *Hin*dIII, and the fragments that migrated to the 4.2-kb region on an agarose gel were cloned into the *Hin*dIII site of pACYC184, which in turn was used to transform strain CE1036 *recA*. Transformants were first selected for Cm^r Km^r. All four transformants thus selected were sensitive to phage Tulb,

FIG. 1. Construction of plasmid pMAN036. ▭, Chromosomal DNA of the *micF-ompC* region. The short and long arrows in the open boxes indicate the coding region and the direction of transcription of *micF* and *ompC*, respectively; broken arrow represents the nonfunctioning *ompC* gene, the upstream region of which has been deleted. ▷ and ▶, Coding region and direction of transcription of *lacZ-lacY* and Km^r, respectively; ——, vector plasmid DNA; ●, temperature-sensitive replicon. The restriction endonucleases used are shown in parentheses (abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I). Cleavage sites are shown. When one enzyme cleaves at more than one site in a plasmid, the sites are distinguished with numbers, e.g., B_1 and B_2. S and L outside parentheses denote small and large fragments, respectively, formed as a result of digestion with the endonuclease. All plasmids were transferred into strain YO160 *recA*, except for pMAN032, pMAN035, and pMAN036, which were transferred into strain MH760 *recA*. Antibiotic resistance genes and plasmid sites are also indicated.

FIG. 4. DNA sequence analysis of the chromosomal *micF* deletion. (A) Strategy for nucleotide sequencing. The *micF-ompC* region on the mutant chromosome which was cloned into pMAN042 is shown. The symbols and abbreviations used are described in the legend to Fig. 1. Hc, *Hin*cII. Small arrows outside the box indicate fragments used for sequencing, with the position of the ^{32}P-labeled 5′ end indicated (●). The broken region of an arrow indicates that the sequence of that region was not determined. (B) Nucleotide sequence around the *micF-ompC* region. The nucleotide sequences determined are underlined. The *micF* region that was deleted and replaced by the Km^r gene-carrying fragment is boxed. Dots between the two strands indicate sequences complementary to *ompF* mRNA. Arrows, Transcriptional initiation sites for *micF* and *ompC* (22). Pribnow boxes (PB) and −35 regions for the *micF* and *ompC* promoters are also indicated (22). A heavy line above the sequences indicates the 120-bp fragment used as a probe for the hybridization analysis (Fig. 3). This fragment was kindly prepared by T. Mizuno. The broken line indicates that the exact location of the terminus was not determined.

which requires the OmpC protein as a receptor. The restriction map of the 4.2-kb *Hin*dIII fragment thus cloned was the same as that of pMAN036 (data not shown). From these results we conclude that the strain SM3001 chromosome indeed carried the 4.2-kb *Hin*dIII fragment of pMAN036. One of the plasmids, pMAN042, was used for DNA sequencing to confirm deletion of the *micF* gene directly. The *Bam*HI-*Bam*HI subfragment of the 4.2-kb fragment, which originated in the Km^r vector plasmid, was removed, and the DNA sequences of the 90-bp *Hin*cII-*Bam*HI and 310-bp *Bam*HI-*Bgl*II subfragments were determined (Fig. 4A). Comparison with the known DNA sequences revealed that the *Hin*cII-*Bam*HI fragment represents sequences downstream from *micF* and the *Bam*HI-*Bgl*II fragment represents the right upstream region from the *ompC* promoter (Fig. 4B), indicating that the promoter region and the region complementary to the *ompF* mRNA of the *micF* gene were almost fully deleted. From all the evidence described above, we concluded that strain SM3001 is a *micF* deletion mutant.

Expression of *ompF* and *ompC* in the *micF* deletion mutant. The *micF* gene codes for a small RNA molecule whose primary structure is complementary to the upstream end of *ompF* mRNA, and the *micF* RNA was proposed to inhibit the translation of the *ompF* mRNA (22). It was also proposed that inhibition by the *micF* RNA is responsible for osmoregulation of *ompF* gene expression. It was therefore expected that the *micF* deletion would cause deregulation of *ompF* expression. Contrary to this expectation, *ompF* gene expression in the *micF* mutant was still osmoregulated normally and was not enhanced (Fig. 5). In fact, expression was slightly repressed. Also unexpectedly, *ompC* gene expression was significantly enhanced, especially at low osmolarity, although it was still osmoregulated. The enhancement of *ompC* gene expression with some repression of *ompF* gene expression in the *micF* mutant will be discussed below.

In any event, it is clear that osmoregulation of *ompF* gene expression was maintained in the *micF* mutant and that the *micF* deletion did not enhance OmpF synthesis. We conclude, therefore, that the presence of the *micF* gene is not crucial for osmoregulation of OmpF synthesis in wild-type *E. coli*.

Effect of *micF* copy number on expression of *ompF*. The *micF* gene cloned into a high-copy-number plasmid represses *ompF* gene expression (22). On the other hand, the present work suggests that a single copy of the *micF* gene on the chromosome does not repress *ompF* gene expression.

FIG. 5. Expression of *ompF* and *ompC* in the *micF* deletion mutant. Strains HO201 (wild-type) and SM3002 (Δ*micF1*) were grown with the indicated concentrations (wt/vol) of sucrose. Triton X-100-insoluble fractions (25 μg of protein) were prepared from cell envelopes and analyzed on polyacrylamide gels. The positions of OmpC, OmpF, and OmpA are indicated.

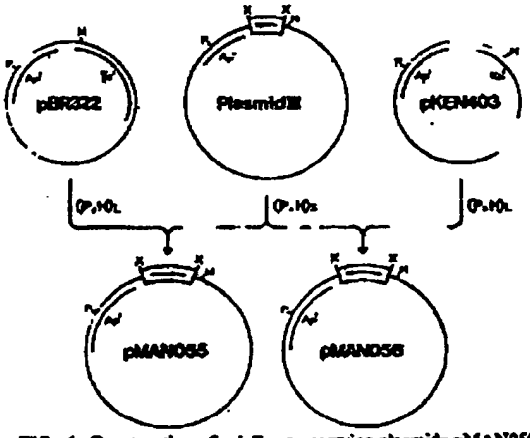

FIG. 6. Construction of *micF* gene-carrying plasmids pMAN055 and pMAN056. In plasmid III (22), the *micF*-carrying CX28 fragment (300 bp ▭) is inserted between the *Xba*I sites (X). The arrow in the box represents *micF*. The 1.0-kb *Pst*I-*Hin*dIII fragment from plasmid III was ligated with the 3.6-kb *Pst*I-*Hin*dIII vector fragment from pBR322 or the 3.8-kb *Pst*I-*Hin*dIII vector fragment from pKEN403 to construct pMAN055 and pMAN056, respectively. The structures of the two plasmids thus constructed were confirmed by DNA restriction analysis. See the legend to Fig. 1 for other abbreviations.

We therefore examined the effect of the *micF* gene cloned into plasmids at different copy numbers. pBR322 and pKEN403 were used as high- and low-copy-number plasmid vectors, respectively. The *micF* gene-carrying fragment was prepared from plasmid III (22) and cloned into pBR322 and pKEN403 to construct pMAN055 and pMAN056, respectively (Fig. 6). The ratio of the copy number of pMAN055 to that of pMAN056 in strain MC4100 was about 5 to 1 (data not shown). Strain MC4100(pMAN055) lost the ability to produce OmpF (Fig. 7, lane 1), whereas in MC4100(pMAN056), no significant repression of OmpF synthesis was observed (Fig. 7, lane 2). The results were the same irrespective of the osmolarity of the medium. The copy number per genome equivalent of pMAN056 was assumed to be about six, since the vector domain of the plasmid was derived from pSC101 (3). It is reasonable to assume, therefore, that a single copy of the *micF* gene on the *E. coli* chromosome is insufficient to repress *ompF* gene expression significantly.

DISCUSSION

Techniques of gene replacement between plasmids and chromosomes via homologous recombination have been developed (8, 14, 28). The techniques require (i) a plasmid that cannot replicate extrachromosomally and (ii) a simple selection method for the plasmid integrate and also one for segregating the plasmid from the plasmid integrate (Fig. 2). A plasmid integrate is a strain in which a direct nontandem duplicate of bacterial sequences separated by vector sequences is integrated into the chromosome via homologous recombination.

The method of gene replacement we have developed in this work is unique in the following points. The use of a plasmid carrying the Km^r and *lac* genes is the major advantage of the present method, especially for the construction of a deletion mutant, since a deletion mutant can be efficiently obtained by one-step selection for Km^r Lac$^-$ on MacConkey plates without knowing the phenotype caused by the mutation. The use of a plasmid carrying the temperature-sensitive replicon that cannot replicate at 42°C is another advantage over the use of a ColE1-like plasmid (8), whose replication depends on the *polA* gene (16), because in the latter case the construction strain cannot be transformed with a plasmid that is unable to replicate in the *polA* background. Our methodology is applicable to the isolation of mutants carrying deletions of any *E. coli* chromosomal gene provided that it is dispensable.

We constructed a *micF* deletion mutant by the procedure described above. The deletion was confirmed by various genetic and biochemical tests, including nucleotide sequence analysis. We also confirmed that the wild-type *E. coli* chromosome, from which the *micF* deletion mutant was constructed, possesses only one copy of the *micF* gene. Contrary to expectation on the basis of the proposed function of *micF*, *ompF* expression was not enhanced and was still osmoregulated normally in the *micF* mutant, indicating that the *micF* gene on the chromosome does not play a critical role in osmoregulation.

It should also be noted that in the *micF* deletion mutant *ompC* gene expression was enhanced, with slight repression of *ompF* gene expression. The promoter regions of *ompC* and *micF* are located next to each other on the *E. coli* chromosome (22). It can be assumed, therefore, that both promoters compete with each other for an RNA polymerase molecule, so that the deletion of one of them enhances expression of the other. This view was supported by recent analyses of expression of the two genes (unpublished data). The decrease in *ompF* expression in the *micF* deletion mutant may be a result of the high expression of *ompC*.

It was reported that the *micF* gene cloned in a high-copy-number plasmid repressed *ompF* expression (22). This result, together with the present work, suggests that the gene dosage would be crucial to the functioning of the *micF* RNA as a repressor. This view was supported by the results presented in Fig. 7, where it can be seen that the *micF* gene cloned in the low-copy-number plasmid did not repress *ompF* gene expression. Taking all the results together, we conclude that although the *micF* gene has the potential to repress *ompF* gene expression and osmoregulation as reported previously (21, 22), one copy of it on the chromosome is insufficient for it to play a critical role in osmoregulation under the conditions we employed in this work.

Recently, Schnaitman and McDonald obtained a deletion mutant lacking the *micF-ompC* region (29). OmpF synthesis in the mutant was partially constitutive; OmpF synthesis is significant in high-osmolarity media. The *micF* deletion

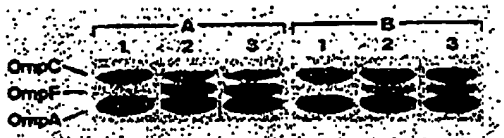

FIG. 7. Effect of the copy number of *micF* on expression of *ompF*. Strains MC4100(pMAN055) (lanes 1), MC4100(pMAN056) (lanes 2), and MC4100 (lanes 3) were grown in (A) medium A and (B) medium A supplemented with 8% (wt/vol) sucrose. Triton X-100-insoluble fractions (25 μg of protein) were prepared from cell envelopes and analyzed on polyacrylamide gels. The positions of OmpC, OmpF, and OmpA are indicated.

mutant we used in the present study was *ompC**. We are presently studying the effects of *ompC* on *micF* function.

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